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**Re-certification of NIST Standard
Reference Material[®] 2372**
Human DNA Quantitation Standard:
The What, the Why and the How

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What is SRM 2372 Human DNA Quantitation Standard?

Genomic DNA prepared to be double-stranded DNA (dsDNA)



Component A: Single-source male

Component B: Multi-source female

Component C: Multi-source male/female mixture

All solubilized in TE⁻⁴ buffer (10mM Tris, 0.1 mM EDTA, pH 8.0)

Certified for spectroscopic traceability in units of decadic attenuation, D_{10} . The D_{10} scale is a measure of absorbance and is traceable to the unit 1.

The conventional conversion factor for aqueous dsDNA is: $1.0 D_{10}$ at 260 nm = 50 ng/ μ L DNA

In March 2012, SRM 2372 was taken off the market and work performed to re-certify the materials

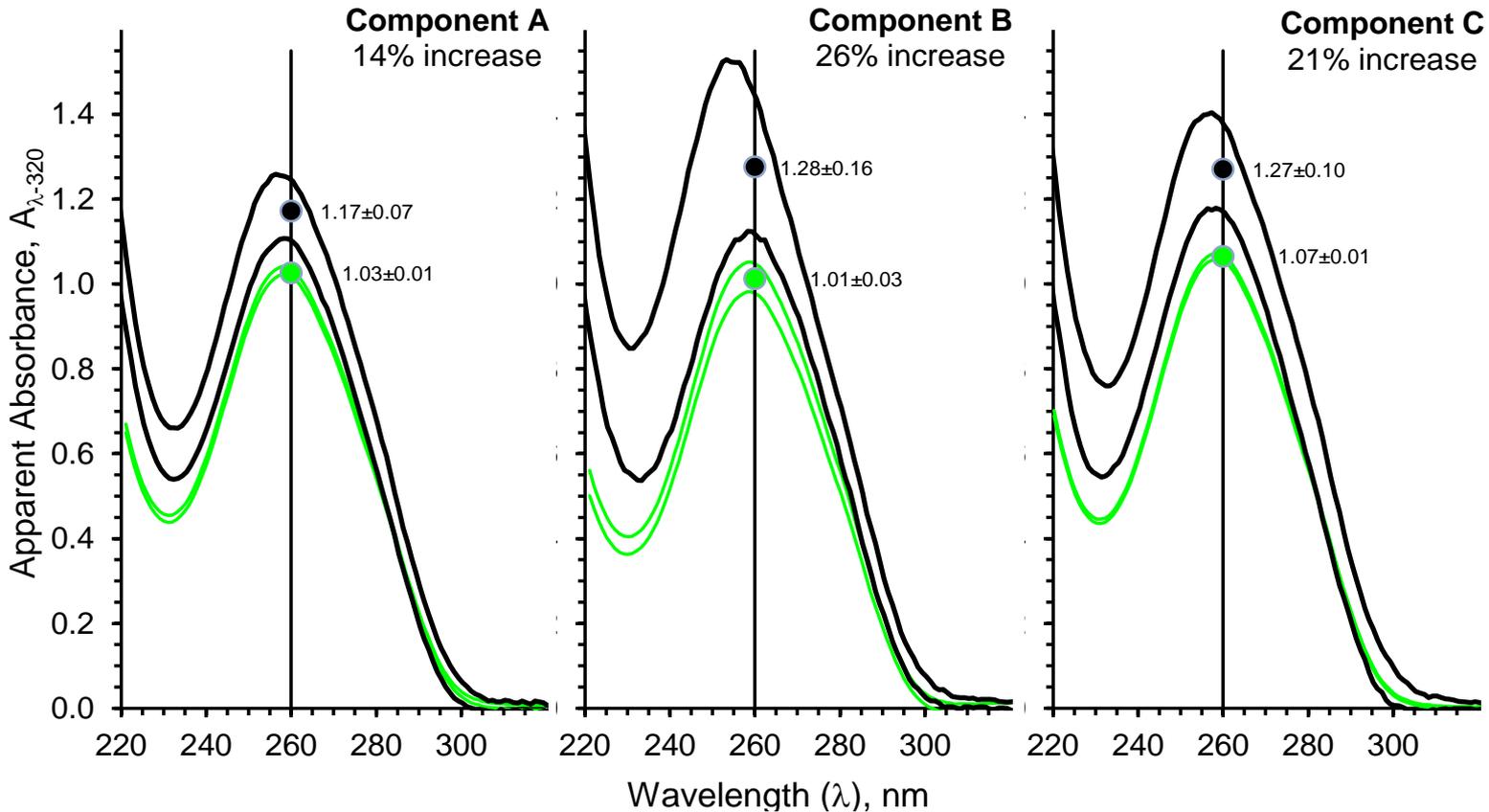
Why Was SRM 2372 Taken Off the Market?

- During measurement of the DNA samples to verify stability of certified values we observed that **the UV absorbance values for the samples had changed significantly**
 - Not due to degradation of the DNA but rather unraveling or opening up of the DNA strands in the TE buffer (**single-stranded DNA absorbs more UV light than double-stranded DNA**)
 - SRM 2372 is certified for UV absorbance (decadic attenuation)
 - One application of this SRM is for calibration of UV spectrophometers
- **The sample changes over time that impact UV absorbance do not appear to affect qPCR sample performance**

Re-certification of SRM 2372 involved forcing the DNA samples into a single-stranded state before measuring UV absorbance

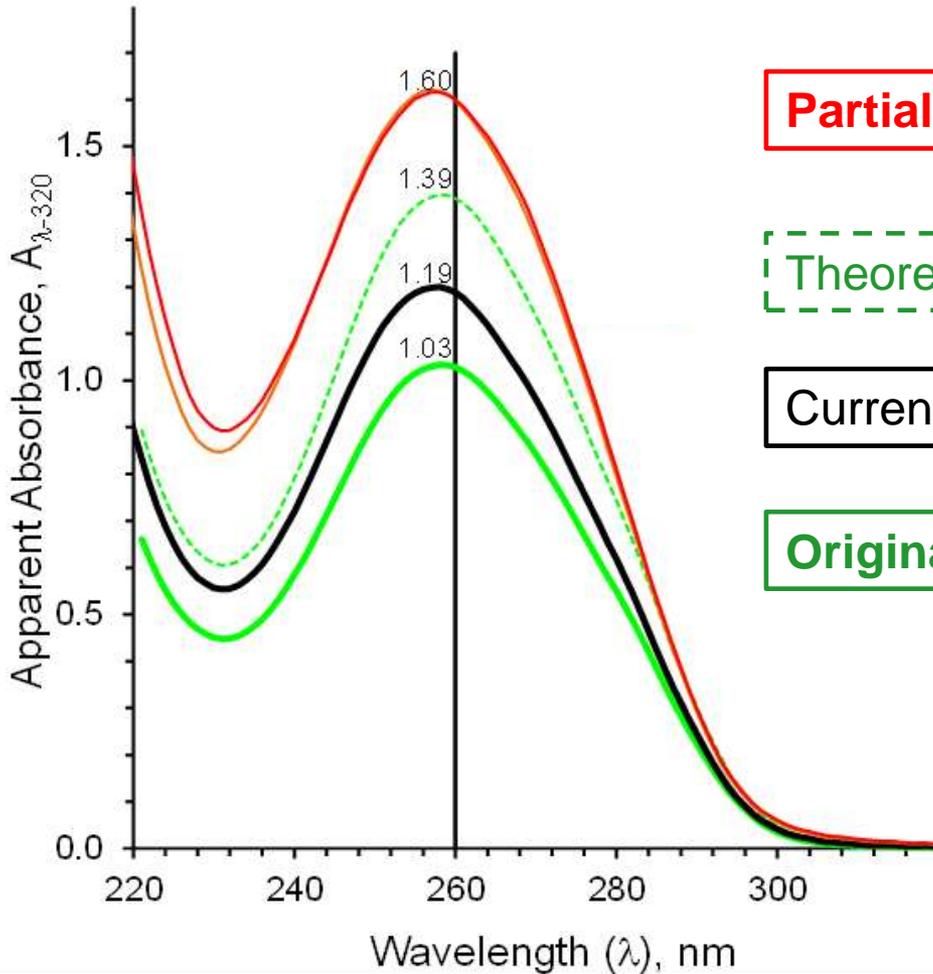
Why did SRM 2372 need to be re-certified?

Six years after production the D_{10} absorbance of these dsDNA solutions had *increased* significantly, suggesting partial conversion to single-stranded DNA (ssDNA)



Green Traces 2006 low/high absorbance spectra
Black Traces 2012 low/high absorbance spectra

Evidence of dsDNA/ssDNA mixture



Partially fragmented DNA profile

Theoretical ssDNA profile

Current average DNA profile

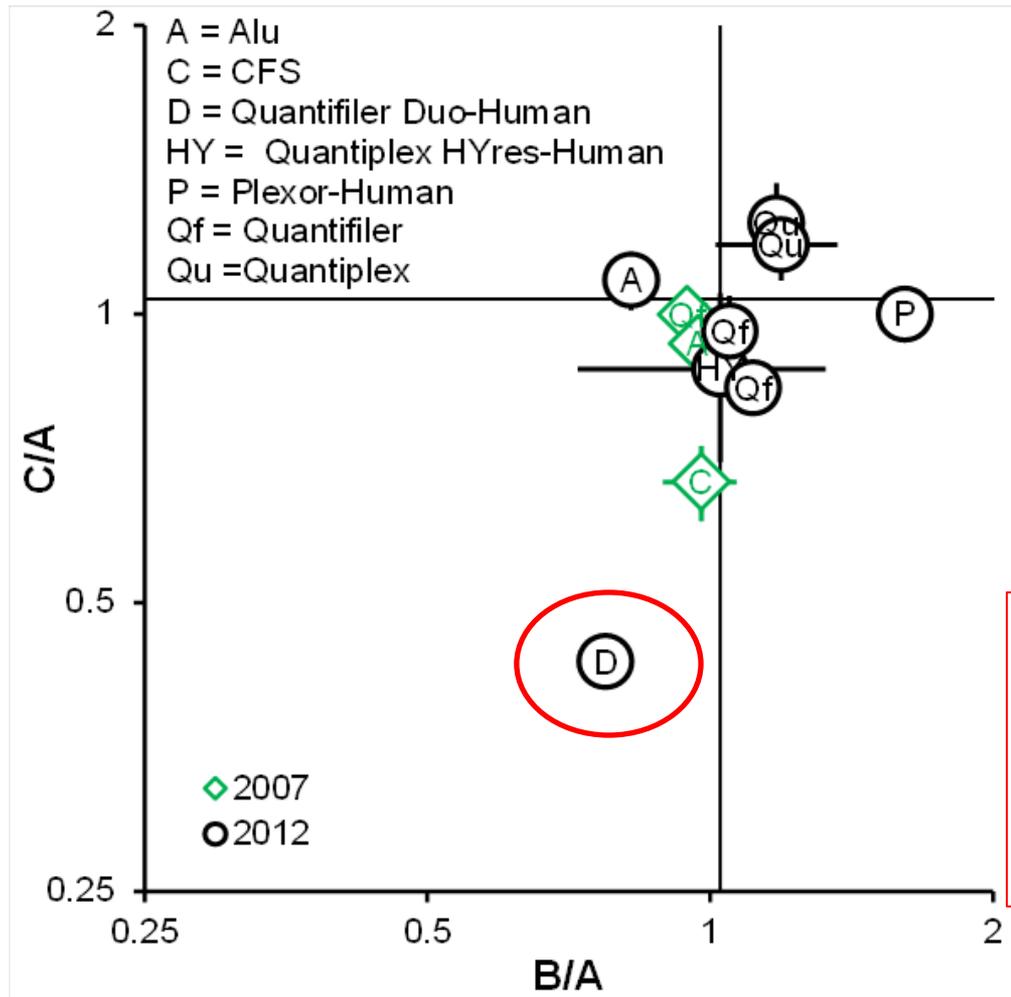
Original dsDNA profile

Conventional Conversion Factors
 dsDNA: $D_{10} (260 \text{ nm}) = 1 \rightarrow 50 \text{ ng}/\mu\text{L}$
 ssDNA: $D_{10} (260 \text{ nm}) = 1 \rightarrow 37 \text{ ng}/\mu\text{L}$
(some references say 40 ng/μL)

Component A

Some customers use SRM 2372 as an absorbance standard

Does ssDNA vs dsDNA affect qPCR?



No

The green diamonds represent assays performed in 2007 while the black circles represent the current results.

Quantifiler Duo was released AFTER 2007 release of SRM 2372

We've previously noted the difference seen with component C and Quant Duo
http://www.cstl.nist.gov/strbase/pub_pres/Promega2009poster.pdf

How did we re-certify SRM 2372?

- **Force the material to an all ssDNA conformation**
- Measurements were made using a modification of ISO 21571 Annex B “Methods for the quantitation of the extracted DNA”
 - Combine equal volumes of the DNA extract and freshly prepared 0.4 mol/L NaOH
 - Measure against a reference of equal volumes of TE⁻⁴ buffer and the 0.4 mol/L NaOH
 - Microvolume spectrometers may have issues with NaOH solutions
- Apparent Absorbance is $D_{10} (260 \text{ nm}) - D_{10} (320 \text{ nm})$

Component A	Component B	Component C
0.777 (0.725 – 0.829)	0.821 (0.739 – 0.903)	0.804 (0.753 – 0.855)

Convert Apparent Absorbance to ng/ μ L

- Conventional concentration values are derived from the assertion that a solution of ssDNA with an absorbance of 1.0 at 260 nm and a pathlength of 1.0 cm has a DNA mass concentration of 37 μ g/mL (37 ng/ μ L)

Parameter	A	B	C
$D_{10} (260 \text{ nm}) - D_{10} (320 \text{ nm})$	1.2	1.3	1.3
2012 DNA Mass Concentration	57	61	59
2007 DNA Mass Concentration	52.4	53.6	54.3
Theoretical difference, %	9 %	14 %	9 %
Theoretical difference, Ct	0.12 cycle	0.19 cycle	0.12 cycle

Difference between the original and re-certified values is within the noise of the assay

Do we measure ng/ μ L or amplifiable targets or accessible amplifiable targets?

- qPCR methods have evolved to try to establish the link between “quality/quantity” of the DNA extract and the resulting STR profiles
- The STR profiles generated are based on the *accessible amplifiable* targets
- We propose using digital PCR (dPCR) to **directly** assess the number of *accessible amplifiable* targets
 - This measurement technique has been shown to work well with plasmid DNA
 - Not yet demonstrated to work with human genomic DNA

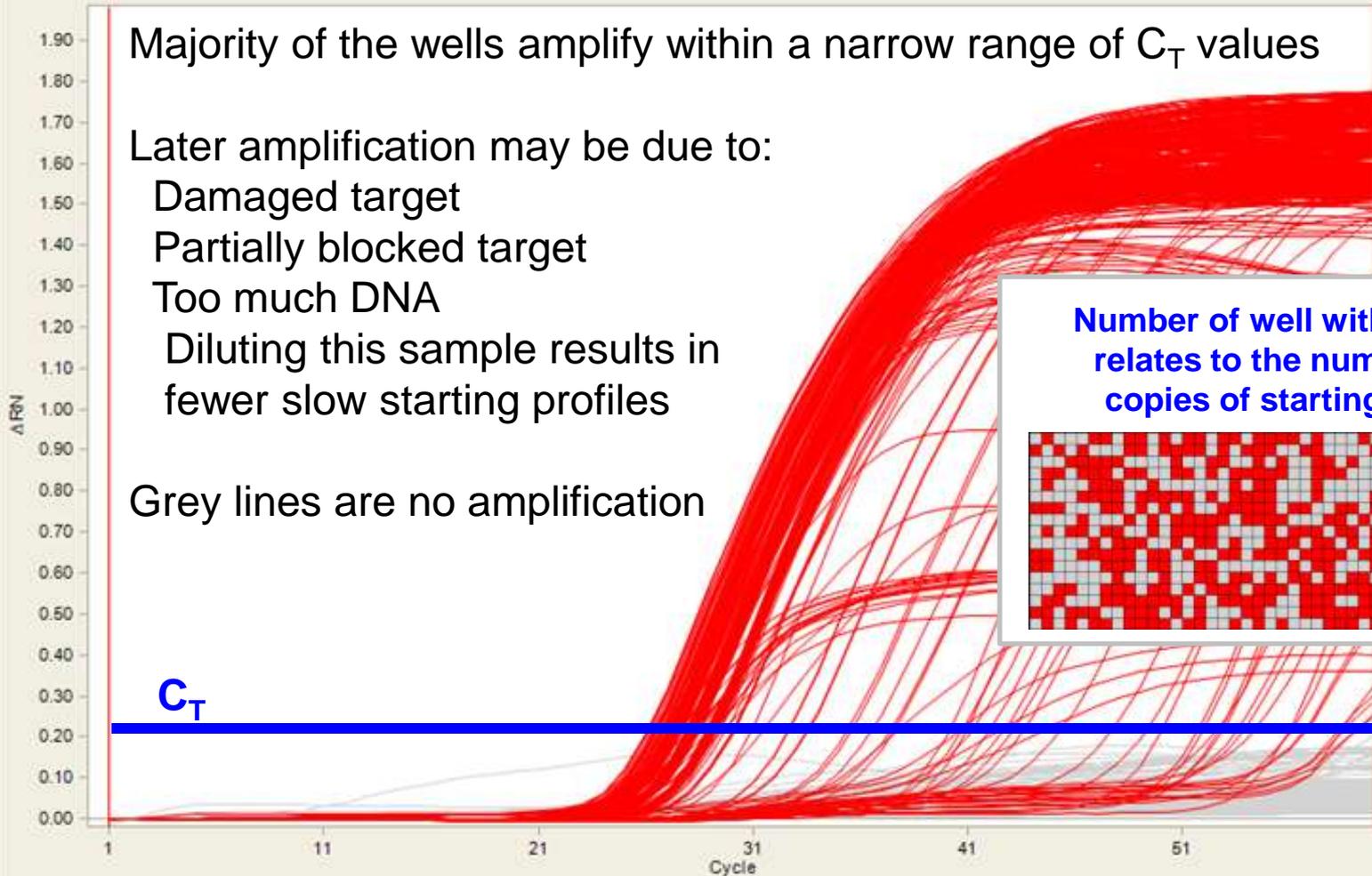
Digital PCR (dPCR) Overview

- Estimates the number of *accessible amplifiable* targets without an external calibrant
- Samples are split into 100s to 1000s of reaction chambers
 - Fluidigm 12.765 Digital Array
 - 765 chambers × 12 panels = **9180 dPCR reactions**
- The count of the number of chambers containing at least 1 target can be used to estimate the total number of targets in a sample

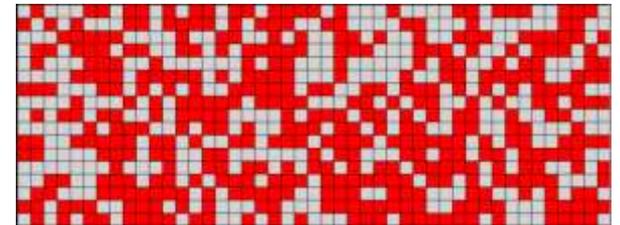


Fluorescent signal as a function of amplification cycle in 765 dPCR reactions

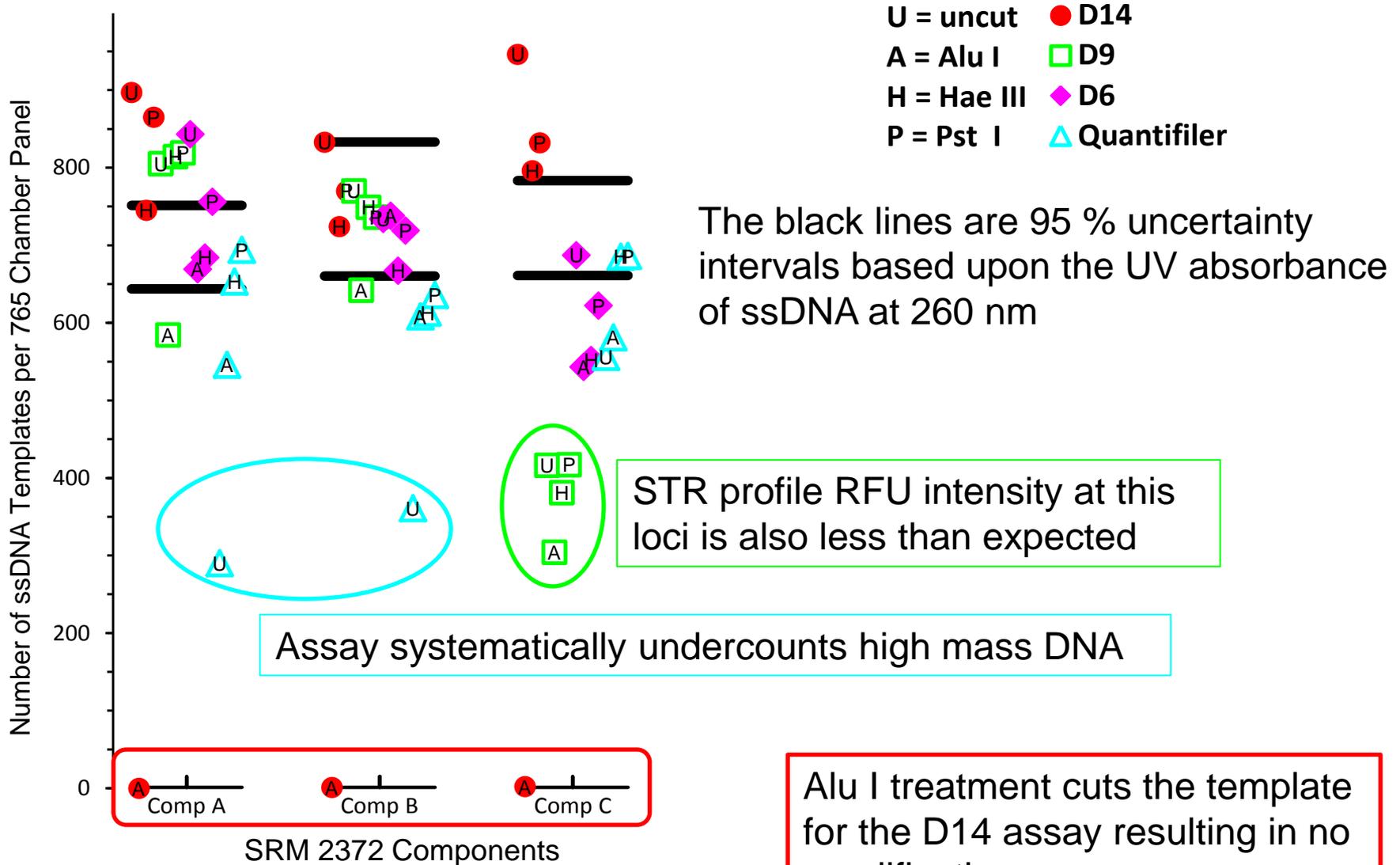
Panel01: Amplification



Number of well with signal relates to the number of copies of starting DNA



Four treatments with four dPCR assays



dPCR is Planned as the Next Certification Method

- The next generation of SRM 2372 will be certified for “copy/target number” not UV absorbance
 - dPCR assays require optimization to improve measurement accuracy and reproducibility
- It is important to realize that there is no one human genomic material that will have the same “target number” for all assays; **lots of variability is being discovered at the genome level in terms of copy number variants and chromosomal rearrangements**

Summary

- NIST SRM 2372 has been re-certified through forcing dsDNA to become ssDNA in order to improve the UV absorbance measurements
- qPCR measurements have not been significantly impacted by the new certified (and DNA concentration) values
- It is important to keep in mind that using DNA quantitation as a gate keeper is impacted by new qPCR targets and STR kit PCR buffer formulations
 - Insensitive qPCR assays may not accurately reflect ability of new, more sensitive STR kits to obtain results

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